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Massachusetts Institute of Technology

Candida albicans Dicer (CaDcr1) is required for efficient ribosomal and spliceosomal RNA maturation

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The generation of mature functional RNAs from nascent transcripts requires the precise and coordinated action of numerous RNAs and proteins. One such protein family, the ribonuclease III (RNase III) endonucleases, includes Rnt1, which functions in fungal ribosome and spliceosome biogenesis, and Dicer, which generates the siRNAs of the RNAi pathway. The recent discovery of small RNAs in *Candida albicans* led us to investigate the function of *C. albicans* Dicer (CaDcr1). CaDcr1 is capable of generating siRNAs in vitro and is required for siRNA generation in vivo. In addition, *CaDCR1* complements a *Dicer* knockout in *Saccharomyces castellii*, restoring RNAi-mediated gene repression. Unexpectedly, deletion of the *C. albicans CaDCR1* results in a severe slow-growth phenotype, whereas deletion of another core component of the RNAi pathway (*CaAGO1*) has little effect on growth, suggesting that *CaDCR1* may have an essential function in addition to producing siRNAs. Indeed CaDcr1, the sole functional RNase III enzyme in *C. albicans*, has additional functions: it is required for cleavage of the 3′ external transcribed spacer from unprocessed pre-rRNA and for processing the 3′ tail of snRNA U4. Our results suggest two models whereby the RNase III enzymes of a fungal ancestor, containing both a canonical Dicer and Rnt1, evolved through a series of gene-duplication and gene-loss events to generate the variety of RNase III enzymes found in modern-day budding yeasts.

Argonaute | *CDL1* | bifunctional dicer

RNA processing plays pivotal roles in ribosome biogenesis (1), mRNA maturation (2), tRNA synthesis (3), and siRNA generation (4). Each requires the accurate and efficient maturation of precursor RNAs to generate specialized RNA molecules designed to perform distinct cellular tasks.

Dicer is the key ribonuclease important for the generation of siRNAs and related types of small RNAs, which in most eukaryotic organisms have key functions in viral defense, transposon silencing, and cellular gene repression (5–10). Dicer generates siRNA duplexes from long double-stranded RNA (dsRNA). One strand of the duplex is loaded into an effector complex containing Argonaute, where it base pairs to target RNAs, thereby directing gene repression during the process of RNAi. Both Dicer and Argonaute are absent in *Saccharomyces cerevisiae* (8) but present in some other budding-yeast species, including *Saccharomyces castellii* and *Candida albicans*, a fungal pathogen (11). The *S. castellii* Dicer (ScaDcr1) and Argonaute (ScaAgo1) are required for gene silencing but are not essential for viability (11). *C. albicans* has a Dicer-like activity that can process dsRNA into small RNAs in vitro, and in vivo produces small RNAs that bear the chemical signature of molecules generated by a ribonuclease III (RNase III) cleavage (11). However, *C. albicans* Argonaute (CaAgo1) and Dicer (CaDcr1) are reported to be insufficient to induce RNAi-mediated gene silencing in *C. albicans* (12). Thus, the in vivo roles of CaDcr1 and CaAgo1 remain a mystery.

Although the domain structure of budding-yeast Argonaute proteins is similar to that of Argonaute proteins found in other eukaryotes, the budding-yeast Dicers are quite dissimilar from their canonical counterparts found in other fungi and higher eukaryotes (11). These canonical fungal Dicers contain two RNase III domains as well as helicase and Piwi Argonaute Zwille (PAZ) domains (13). By contrast, ScaDcr1 and CaDcr1 have only a single RNase III domain adjacent to a dsRNA-binding domain (dsRBD) and an additional C-terminal dsRBD (11) (Fig. 1*B*). Despite significant differences in domain structure between the budding-yeast and canonical Dicers, the RNase III signature motif and active-site residues important for dsRNA cleavage are conserved in ScaDcr1 and CaDcr1, suggesting that the RNase III cleavage activity is conserved in ScaDcr1 and CaDcr1 (14–16).

Bacteria and some budding yeasts encode a single protein with this RNase III domain, whereas higher eukaryotes often encode several RNase III enzymes that perform distinct tasks in RNA metabolism. Rnt1, the sole RNase III protein in *S. cerevisiae*, plays crucial roles in ribosomal and spliceosomal RNA maturation (17–20). For example, *S. cerevisiae* Rnt1 (SceRnt1) cleaves the 3′ external transcribed spacer (ETS) hairpin from primary 35S rRNA transcript, thereby permitting downstream rRNA processing events (20). Mutants lacking SceRnt1 function grow slowly because they have a severe defect in processing the nascent 35S rRNA transcript, resulting in decreased ribosome biogenesis (20, 21). *S. castellii* encodes two RNase III proteins, ScaRnt1 and ScaDcr1, each thought to perform specialized functions in RNA processing (11). ScaRnt1 has the same domain structure as SceRnt1 and presumably plays analogous roles in ribosomal and spliceosomal RNA maturation. ScaDcr1 contains a second dsRBD not found in SceRnt1 (Fig. 1*B*), suggesting that this domain functions in Dicer-specific activities as seen with *Kluyveromyces polysporus* Dcr1 (16). The in vivo roles for *C. albicans* RNase III homologs are unknown.

To better understand the roles of Dicer and Argonaute in *C. albicans*, we analyzed the consequences of deleting the genes encoding each of these proteins. *C. albicans* mutants lacking *AGO1* grow normally. We were only able to knock out both endogenous copies of *DCR1* when *DCR1* was expressed *in trans* from an inducible promoter. Analysis of strains defective in *DCR1* show that this protein is unusual: *DCR1* encodes a versatile RNase III enzyme that acts as both a Dicer, catalyzing the production of siRNAs, and as an rRNA and snRNA processing enzyme (cleaving the 3′ ETS from unprocessed pre-rRNA and processing the 3′ tail of snRNA U4) like the *S. cerevisiae* Rnt1 enzyme. The Rnt1-like roles of CaDcr1 are likely responsible for the severe slow growth phenotype of mutant strains lacking *DCR1*. Our data suggest that the RNase III enzymes of fungi have evolved through a number of

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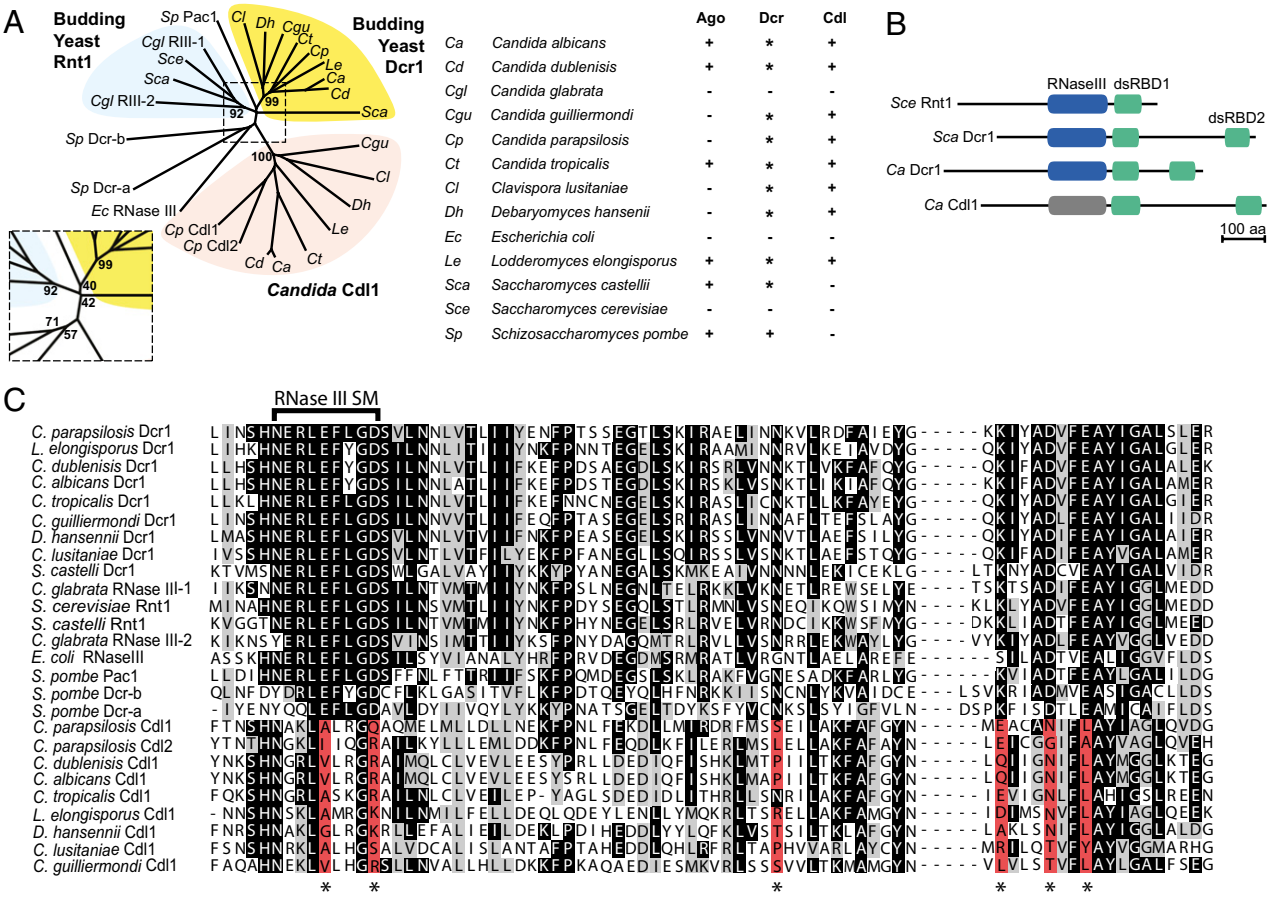

Fig. 1. (*A*) Phylogenetic relationship between RNase III enzymes of indicated species. Bootstrap values for key nodes are shown, with central region (boxed) enlarged for clarity. Table indicates the presence (+) or absence (-) of the indicated gene. For Dicer, (*) designates budding-yeast Dicer, whereas + indicates canonical Dicer. (*B*) Domain structure of budding yeast RNase III enzymes. (*C*) Sequence alignment around the RNase III catalytic residues from the protein sequences from *A*. Sequence conservation is labeled in grayscale, with the most highly conserved residues most darkly colored. The six residues (corresponding to SceRnt1 E278, D282, N315, K346, D350, and E353) altered to catalytically inactive residues in the Cdl1 (*Candida dicer-like)* protein family are labeled (*) and shaded red when inactive. The 9-aa RNase III signature motif is labeled. ClustalW 1.81 with default setting was used to generate both the phylogenetic tree and the sequence alignment.

gene-duplication, neofunctionalization, subfunctionalization, and gene-loss events.

Results

***C. albicans AGO1* and *DCR1* Mutants.** To characterize the in vivo roles of CaDcr1 and CaAgo1, we attempted to construct homozygous deletions of *DCR1* and *AGO1*. The homozygous deletion strains of *AGO1* (*ORF19.2903*) were constructed from wild-type strains by successive transformations with the same deletion construct, with marker removal in between as described in *Materials and Methods*. Homozygous *AGO1* deletion in two different *C. albicans* strain backgrounds had no effect on growth rate (Fig. S1), germ tube formation, or colony morphology.

A search of the *C. albicans* genome for homologs of *S. castellii* Dcr1 revealed two ORFs predicted to encode RNase III domains. One previously identified as the *Candida* Dicer ortholog is encoded by *ORF19.3796* (11), and another is encoded by *ORF19.3773.* Both of these genes are conserved throughout the *Candida* clade and have the same domain structure as ScaDcr1 (Fig. 1 *A* and *B*). Alignment of all of the RNase III domains from the *Candida* clade divided the RNase III-containing proteins into two families, *ORF19.3796* homologs and *ORF19.3773* homologs (Fig. 1 *A* and *C*). Examination of the *ORF19.3773* sequence revealed that amino acids corresponding to E278, D282, N315, K346, D350, and E353 of SceRnt1, which are highly conserved residues involved in metal-ion coordination at the active sites of RNase III enzymes, are altered to residues likely to inactivate RNase activity (Fig. 1*C*) (16). On the basis of these observations, *ORF19.3796* has been named *DCR1*, and *ORF19.3773* has been named *CDL1*, *Candida* Dicer-like.

Deletions of one copy of *DCR1* in *C. albicans* were constructed; however, deletions of the second copy could not be recovered. Instead, transformations designed to generate a homozygous deletion of *DCR1* resulted in transformants with various genome abnormalities (including aneuploidy and local genome rearrangement events)—all of which retained a functional copy of *DCR1* when examined by Southern blot. On the assumption that complete loss of Dcr1 function might be lethal, we constructed a strain with an ectopic copy of either a maltose-inducible *DCR1* at the *ENO1* locus or a doxycycline (Dox)-inducible *DCR1* at the *ADH1* locus, and one deleted copy of *DCR1.* Under conditions that permitted ectopic *DCR1* expression, we could isolate strains containing disruption of both copies of *DCR1* (*Δdcr1/Δdcr1*).

The *Δdcr1/Δdcr1* strains constructed in two different backgrounds had a severe slow-growth phenotype on both solid and liquid media that did not permit transgene expression (Fig. 2 and Fig. S2 *A–N*). The inability to delete both copies of *DCR1* without supplementing the activity from a transgene, coupled with the slow

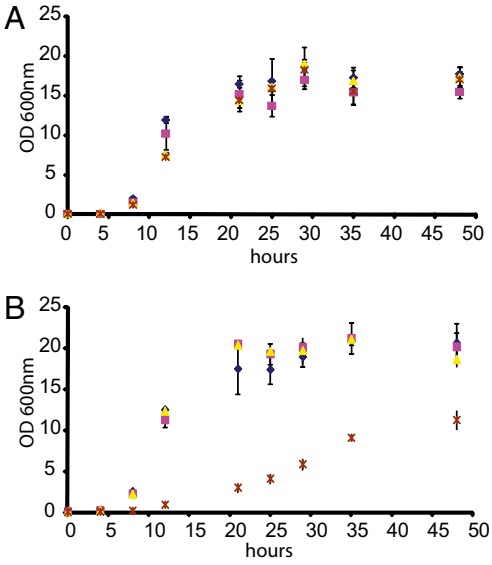

Fig. 2. Effects of *DCR1* on *C. albicans* growth. (*A*) WT [BWP17 (◆)], *dcr1/DCR1* [DAB151 (▲)], *dcr1/DCR1 ENO1/eno1::MAL2p-DCR1* [DAB223 (■)], and *dcr1/dcr1 ENO1/eno1::MAL2p-DCR1* [DAB225 (×)] grown on a roller drum in synthetic complete (SC) maltose. (*B*) Strains from *A* grown in SC glucose. OD_{600} was used to measure growth. Points plotted are the average ODs of three replicates, and error bars represent 1 SD of the mean.

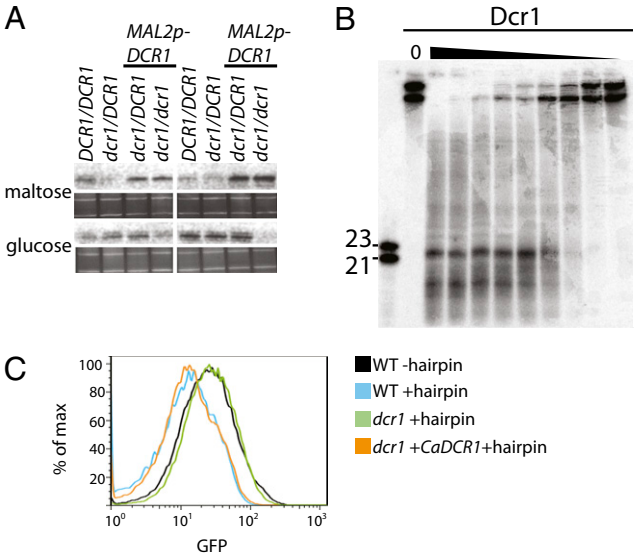

Fig. 3. Functions of *DCR1* in vivo and in vitro. (*A*) Small RNA Northern blot analysis of RNA purified from cells grown overnight in SC and resolved on a denaturing gel. Strains are of indicated genotype, and probe was Northern Primer 1. *Left*: BWP17 background; *Right*: CAI4 background. Below each blot, ribosomal RNA has been visualized with ethidium bromide to show loading. (*B*) In vitro processing of radiolabeled dsRNA by recombinant *C. albicans* Dcr1. Products were resolved on a denaturing gel. Leftmost lane contains size standards, and second lane contains substrate with reaction buffer and no Dcr1. (*C*) *C. albicans DCR1* complements *S. castellii dcr1* for silencing of *GFP*. Strains DPB331 (black), DPB333 (blue), and DPB339 (green) were transformed with pV401, and DPB339 was also transformed with pV406 (orange). Cells were grown in synthetic galactose media and subjected to FACS analysis. Histogram plots represent GFP values for 30,000 cells.

growth of deletion strains in which the transgene was not induced, suggest that *C. albicans DCR1* encodes an essential function. The slow growth of strains under repressed conditions might be attributable either to the incomplete repression of the ectopic copy of *DCR1* or to residual Dcr1 protein.

***C. albicans* Dcr1 Generates Small RNAs in Vivo and in Vitro.** The construction of a strain lacking Dcr1 activity enabled us to determine whether *DCR1* function was required to generate siRNAs. Northern analysis of total RNA prepared from cells grown under repressive conditions revealed that two of the most abundant siRNAs in *C. albicans* (11) were significantly lower in *MAL2p-DCR1, Δdcr1/Δdcr1* than in strains expressing *DCR1* (Fig. 3*A* and Fig. S3 *A* and *B*). Growth of *MAL2p-DCR1, Δdcr1/Δdcr1* in media containing maltose restored siRNA production and actually increased the levels of these siRNAs (Fig. 3*A* and Fig. S3 *A* and *B*) over those found in wild-type cells. Furthermore, a lower abundance siRNA, previously identified by high-throughput sequencing, was only detectable by Northern blotting in *MAL2p-DCR1* containing strains grown on maltose (Fig. S3 *C* and *D*). The increase in siRNA production in the maltose-inducible strains when grown in maltose was likely attributable to increased Dcr1 levels as a result of increased promoter strength, suggesting that Dcr1 is normally limiting for siRNA production. Homozygous deletion of *AGO1 (Δago1/Δago1)* had no effect on siRNA accumulation as tested by Northern blotting (Fig. S3*E*).

Because *Δdcr1/Δdcr1* mutants had diminished siRNA levels in vivo, we sought to determine whether purified Dcr1 had in vitro dicing activity. Recombinant Dcr1 was purified from *Escherichia coli* and incubated with a 500-bp radiolabeled dsRNA substrate. Purified Dcr1 cleaved the substrate to 22-nt products (Fig. 3*B*), a size matching that of small RNAs sequenced from *C. albicans* (11). These results show that purified CaDcr1 is capable of making siRNAs in vitro and are in agreement with previous work showing that *C. albicans* crude extracts can generate siRNAs from an exogenous dsRNA substrate (11).

These results raised the question of whether CaDcr1 can act in the RNAi pathway to silence a gene in vivo. Because a gene-silencing system does not yet exist for *C. albicans*, we tested whether CaDcr1 can mediate silencing in *S. castellii*. ScaDcr1 is required to generate the siRNAs that direct gene silencing (11). A recoded *CaDCR1* was transformed into an *S. castellii dcr1* mutant that expresses green fluorescent protein (*GFP*) from the *URA3* promoter and a hairpin against *GFP* from the *GAL1* promoter. The *CaDCR1* complemented the *S. castellii dcr1* mutant, restoring silencing to levels observed in an *S. castellii* strain with a functional *S. castellii DCR1* (Fig. 3*C*). Together, these in vitro and in vivo data demonstrate that *C. albicans DCR1* encodes a gene with Dicer function.

***C. albicans DCR1* Is Required for rRNA Processing.** The requirement of functional CaDcr1 but not CaAgo1 for normal growth suggests that CaDcr1 might have additional roles in *Candida*. In other fungi, mutants lacking either Dicer or Argonaute have minimal effects on growth rates (11, 22, 23). One possibility is that CaDcr1 functions like the SceRnt1 of *S. cerevisiae*, which plays a critical role in the initial steps of ribosome biosynthesis. Decreased expression of SceRnt1 leads to a severe slow-growth phenotype resembling that observed in the *C. albicans Δdcr1/Δdcr1* mutant. Furthermore, the only additional gene in the *Candida* genome that contains an RNase III domain, *CDL1*, is unlikely to have catalytic activity, which leaves CaDcr1 as the only RNase III with the potential to cleave the ribosomal RNA precursor. We performed Northern analysis of *Δdcr1/Δdcr1 MAL2p-DCR1* to compare rRNA processing of cells grown in glucose or permissive media. Strains expressing *DCR1* had no detectable levels of unprocessed rRNA transcripts, whereas the *Δdcr1/Δdcr1* strains grown in restrictive conditions accumulated unprocessed rRNA

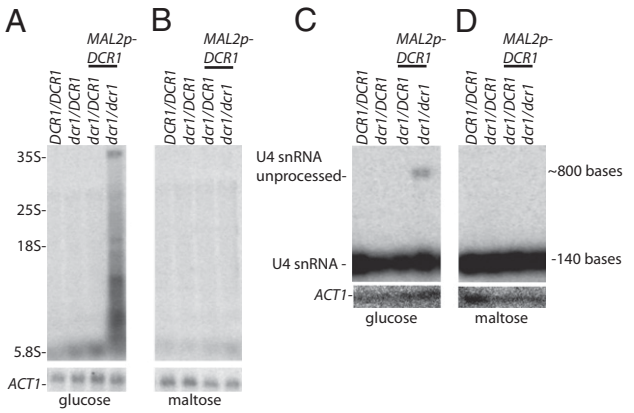

Fig. 4. *DCR1* deletion impact on snRNA and rRNA processing in *C. albicans*. Ten micrograms total RNA prepared from BWP17 background strains grown in indicated carbon source were subjected to Northern analysis probing with pre-rRNA Northern (*A* and *B*), pre-U4 (*C* and *D*), or ACT1 Northern primer (*A*–*D*). Pre-rRNA Northern primer location is depicted in Fig. S5. The pre-rRNA Northern primer hybridizes to the region 21–41 bases past the end of *RDN25*, as depicted in Fig. S5.

transcripts (Fig. 4*A* and Fig. S4 *A*, *C*, and *E*). By contrast, all four strains grown in permissive media had undetectable levels of unprocessed rRNA transcripts (Fig. 4*B* and Fig. S4 *B*, *D*, and *F*). Homozygous deletion of *AGO1* had no effect on rRNA processing (Fig. S4*I*). The rRNA processing defect was unlikely to be an indirect consequence of the loss of an essential gene function, because strains lacking another essential gene (*PES1* under control of *MAL2p*) had severely reduced growth on glucose but did not display the same rRNA processing defect we observed in Δ*dcr1*/Δ*dcr1 MAL2p-DCR1* (Fig. S4*J*).

Because *S. cerevisiae* Rnt1 plays an additional role in the proper maturation of some snRNA transcripts, we determined whether CaDcr1 also plays an analogous role in snRNA processing. In *S. cerevisiae*, *rnt1* cells accumulate unprocessed U4 transcript (24). On a growth medium containing glucose, we observed an accumulation of unprocessed U4 snRNA transcript in Δ*dcr1*/Δ*dcr1 MAL2p-DCR1* (Fig. 4*C*) but not in *DCR1*/*DCR1*, Δ*dcr1*/*DCR1*, or Δ*dcr1*/*DCR1 MAL2p-DCR1* (Fig. 4*D*), indicating that CaDcr participates in U4 snRNA processing in vivo. Correctly processed U4 snRNA also accumulated in nonpermissive conditions, a result similar to that obtained in *S. cerevisiae* (24). This result suggests that *C. albicans* encodes other proteins able to act in a functionally redundant manner, or the small amount of expression in glucose is sufficient for detectable processing.

Discussion

Our findings suggest that CaDcr1, which seems to be the only active RNase III enzyme in *C. albicans*, is versatile, possessing a dicing function that generates siRNAs and additional functions involved in ribosome and spliceosome biogenesis. We attribute the slow-growth phenotype of Δ*dcr1*/Δ*dcr1* to its ribosome-maturation defects because loss of *CaAGO1* did not affect the growth rate. Knockouts of *S. cerevisiae RNT1* and its *Schizosaccharomyces pombe* homolog *PAC1* also result in severe growth defects that are likely attributable to ribosome-maturation defects similar to those observed in Δ*dcr1*/Δ*dcr1* (21, 25, 26).

The dual function of the *C. albicans* Dicer is unique among the fungal Dicers that have been analyzed. An *S. castellii* strain lacking Dcr1 function resulted in loss of siRNAs but no obvious loss in viability or discernible phenotype related to growth or morphology (11). The only phenotype that has been noted is the loss of RNAi leads to a compatibility with dsRNA killer viruses (27). Previous work identified small RNAs from *C. albicans* Zorro L1 elements, suggesting a role in genome defense. Because the Δ*dcr1*/Δ*dcr1* strain has poor viability, the biological roles of siRNAs and other components of the RNAi pathway were best queried using the Δ*ago1*/Δ*ago1* strain. Although the siRNAs derived from Zorro L1-like elements imply that these transposable elements are silenced by the RNAi pathway (11), we did not observe any obvious difference in Zorro transposition between *AGO1*/*AGO1* and Δ*ago1*/Δ*ago1* strains. However, the Zorro transposition frequency is very low in *Candida*, and slight differences would not have been detected (28).

Our results together with previous work provide insight into the evolution of the budding-yeast RNase III enzymes. Four types of RNase III-like proteins exist in budding yeast (Fig. 1*B*). The *S. cerevisiae* genome encodes a single RNase III enzyme, Rnt1, that is essential for ribosome biosynthesis and also functions in generating spliceosomal RNAs (17, 19, 20), but SceRnt1 does not process dsRNA into siRNAs, consistent with the lack of RNAi in this species (11). *S. castellii* contains two functional RNase III enzymes, one of which likely plays a similar role to SceRnt1 in ribosome and snRNA processing and another, ScaDcr1, which generates siRNAs that guide RNA silencing (11). *K. polysporus* and *Saccharomyces bayanus* both contain syntenic homologs of the *S. castellii DCR1* and *RNT1*. *C. albicans* encodes two proteins with an RNase III domain: Dcr1, which is multifunctional and plays roles in siRNA, snRNA, and rRNA maturation; and Cdl1, which is unlikely to have cleavage activity and whose in vivo role remains a mystery. These two genes are not syntenic with either *DCR1* or *RNT1* from non-*Candida* budding yeasts.

The domain structure of Cdl1 is identical to that of CaDcr1 (Fig. 1*B*), suggesting that it could have originated from a recent gene-duplication event. However, Cdl1 is unlikely to have canonical RNase III activity, because six residues important for RNA cleavage and conserved in other eukaryotic RNase III enzymes are not conserved in Cdl1 homologs. Retention of Cdl1 in the *Candida* clade and conservation of other amino acids in the RNase III domain suggest that it has a function. Catalytically inactive enzyme homologs are common in signaling pathways, and these inactive proteins play roles regulating those pathways (29). Because RNase III proteins act as obligate dimers (14), the potential formation of a heterodimeric complex between CaDcr1 and CaCdl1 is intriguing.

Uncertainties in the branching order within the budding yeast RNase III phylogeny, combined with the absence of informative syntenic relationships, make it difficult to posit a single model that best explains the evolutionary paths of these enzymes. Therefore, we offer two models consistent with our data (Fig. 5). Both assume an ancestral species that had a canonical Dicer (*DICER*) and *RNT1*, as currently observed in the fission yeast *S. pombe*. Model 1 suggests *RNT1* duplication with neofunctionalization to generate a non-canonical Dicer gene (*DCR1*) in a transitional species. The loss of *DICER* left both *RNT1* and *DCR1*, as in present-day *S. castellii*. Loss of *DCR1* and the rest of the RNAi pathway in many budding-yeast lineages (16) left these lineages with only *RNT1*, as observed in present-day *S. cerevisiae* and other members of the *Saccharomyces* complex that lack Argonaute and Dicer homologs. Meanwhile, neofunctionalization of *DCR1* in the *S. castellii*-like ancestor of the *Candida* clade led to the multifunctional enzyme *RNT1*/*DCR1*. *RNT1* loss (and *CDL1* gain through *RNT1*/*DCR1* duplication with neofunctionalization) then generated the genes of *C. albicans*. Model 2 posits early neofunctionalization of an ancestral *RNT1* to generate a transitional species with the multifunctional *RNT1*/*DCR1*. Subsequent *DICER* loss then generated the *Candida*-like budding yeast ancestor, which gained *CDL1* through *RNT1*/*DCR1* duplication with neofunctionalization in the *Candida* lineage. Meanwhile, *DCR1*/*RNT1* duplication with subfunctionalization generated the two RNase III enzymes present in *S. castellii*. This duplication probably did not occur during the whole-genome duplication (WGD) because the synteny typical of paralogs created

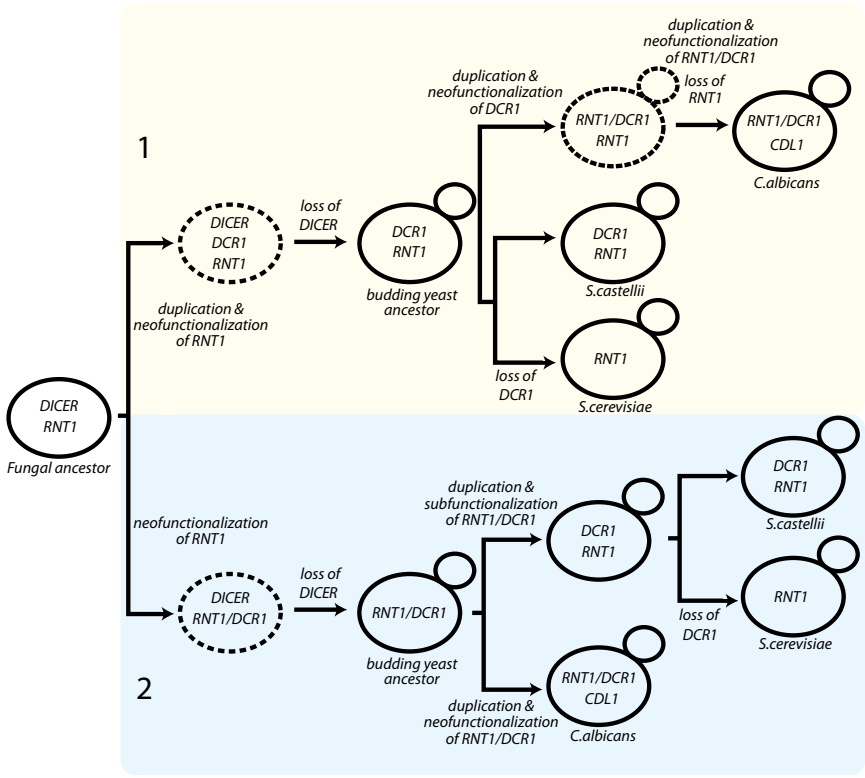

Fig. 5. Two models to explain the evolution of budding yeast DCR1 and RNT1. DICER, canonical Dicer as found in *S. pombe*; DCR1, budding yeast Dicer as found in *S. castellii*, *K. polysporus*, and *S. bayanus*; RNT1, Ribonuclease III as in *S. cerevisiae*; RNT1/DCR1, a multifunctional Dicer found in *C. albicans*; CDL1, *Candida* Dicer-like from the *Candida* clade.

at the WGD, such as that observed between *Candida glabrata RNT1* paralogs, is not observed for *DCR1* and *RNT1*. The ScaDcr1 RNase III domain is not significantly more similar to the RNase III domains of ScaRnt1 or CaDcr1 (Rnt1/Dcr1), thus we cannot distinguish between these two models with confidence. We favor model 1, because model 2 posits gain and then loss of the second dsRBD during the transitions from *RNT1* to *RNT1/DCR1* and back to *RNT1*. This objection would be mitigated in a hybrid of models 1 and 2, in which

Table 1. Yeast strains

Name	Genotype	Parent	Reference
SC5314	WT clinical isolate	—	35
CAI4	ura3Δ::λimm434 ura3Δ::λimm434	SC5314	36
BWP17	*ura3Δ::λimm434 his1::hisG arg4::hisG* *ura3Δ::λimm434 his1::hisG arg4::hisG*	RM1000	37
VY519	*agο1::FRT/AGO1*	SC5314	This study
VY529	*agο1::FRT/agο1::Nat-flp*	VY519	This study
VY561	*agο1::FRT/agο1::FRT*	VY529	This study
VY523	*agο1::FRT/AGO1*	BWP17	This study
VY537	*agο1::FRT/agο1::FRT*	VY523	This study
VY565	*dcr1::FRT/DCR1, adh1::Tetp-DCR1/ADH1*	DAB151	This study
DAB151	*dcr1::FRT/DCR1*	BWP17	This study
DAB157	*dcr1::FRT/DCR1*	CAI4	This study
DAB184	*dcr1::FRT/dcr1-1 adh1::Tetp-DCR1*	DAB151	This study
DAB196	*dcr1::FRT/dcr1-1 adh1::Tetp-DCR1*	DAB157	This study
DAB204	*dcr1::FRT/dcr1-1 adh1::adh1/Tetp-DCR1*	DAB196	This study
DAB232	*dcr1::FRT/dcr1-1 adh1::Tetp-DCR1/ADH1*	DAB184	This study
DAB223	*dcr1::FRT/dcr1-1 eno1::MAL2p-DCR1/ENO1*	DAB151	This study
DAB224	*dcr1::FRT/dcr1-1 eno1::MAL2p-DCR1/ENO1*	DAB157	This study
DAB225	*dcr1::FRT/dcr1-1 eno1::eno1/MAL2p-DCR1*	DAB223	This study
DAB228	*dcr1::FRT/dcr1-1 eno1::eno1/MAL2p-DCR1*	DAB224	This study
JLK713	*pes1::FRT/FRT-MAL2p-PES1*	C665	38
DPB333	*WT + GFP + hairpin*		11
DPB339	*dcr1 + GFP + hairpin*		11

RNT1 is duplicated before *RNT1/DCR1* neofunctionalization and then retained in all ancestors of the *Saccharomyces* lineage. Regardless of the model, the evolution of the budding-yeast RNase III enzymes was not a simple linear path but instead required several instances of gene duplication/loss, often with neofunctionalization/subfunctionalization, to arrive at the diversity currently observed.

Materials and Methods

Dicing Assay. Radiolabeled dsRNA (140 pM) was incubated with purified CaDcr1 protein for 30 min at 37 °C, as described previously (11).

Phylogenic Analysis. RNase III domains were identified using the Conserved Domain search of the National Center for Biotechnology Information (30). Alignments of RNase III domains from selected fungi were created with ClustalW on the Biology Workbench [workbench.sdsc.edu (31)], using default parameters. A tree with bootstrap values was created with Clustaltree on Biology Workbench, and Treeview (32). Synteny analysis was performed using the Yeast Gene Order Browser (33) and *Candida* Gene Order Browser (34).

FACS. Cells were grown overnight in appropriate media, diluted in PBS, and subjected to flow cytometry on a BD FACSCalibur with data acquisition using BD CellQuest Pro. Flow cytometry data analysis was done using FlowJo (Treestar).

Strain Construction. All *Candida* strain genotypes in Table 1 were verified by Southern analysis. The KpnI/SacI fragment from plasmid DAB124 was transformed into *C. albicans* strains BWP17 and CAI4 and selected on yeast extract, peptone dextrose media with Nourseothricin (YPD + Nat). Correct integrants were grown in Difco yeast carbon base with bovine serum albumin (YCB + BSA) to induce expression of the flip recombinase and subsequent removal of the NAT^R gene from the genome generating DAB151 and DAB157. DAB151 and DAB157 were transformed with pV397 integrating *Tetp-DCR1* at the *ADH1* locus. Correct integration in strains DAB151 and DAB157 generated DAB184 and DAB196, respectively. We also transformed DAB151 and DAB157 with pV434 integrating *MAL2p-DCR1* at the *ENO1* locus. Correct integration in strains DAB151 and DAB157 generated DAB223 and DAB224, respectively. A KpnI/SacI fragment of DAB126 was transformed to disrupt the second endogenous copy of *DCR1*, generating *dcr1-1*. The transformation of DAB196 and DAB184 generated DAB204 and DAB232, respectively. An analogous strategy was used to generate strains DAB225 and DAB228 from DAB223 and DAB224, using plasmid DAB177. Although the construction of *DAB225* from *DAB223* resulted in the subsequent loss of the *URA3*, the construction of the Dox-driven expression system resulted in auxotrophically matched strains. Because we see a similar slow-growth phenotype in both constructions, the slow growth of the maltose-inducible strains is not due to the rescue of the uracil auxotrophy. Strains BWP17 and SC5314 were transformed with either pV308 or pV307 (respectively) digested with KpnI/SacII. Correct integrants were grown on YCB + BSA medium to induce expression of FLP, leaving only FRT sites, resulting in strains VY523 and VY519. These strains were subjected to an additional round of transformation and flipping out, resulting in strains VY537 and VY561. *S. castellii* strains DPB333 and DPB339 were transformed using the lithium acetate method, with either pV401 or pV406 for complementation analysis, and selected on YPD + Nat.

Additional methods included in *SI Materials and Methods*.

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